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Drosophila miR-932 modulates hedgehog signaling by targeting its co-receptor Brother of ihog

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ABSTRACT

Hedgehog (Hh) proteins act as morphogens in a variety of developmental contexts to control cell fates and growth in a concentration-dependent manner. Therefore, secretion, distribution, and reception of Hh proteins must be tightly regulated and deregulation of these processes contributes to numerous human diseases. Brother of ihog (Boi) and its close relative Ihog (Interference hedgehog) are cell surface proteins that act as Hh co-receptors required for Hh signaling response and cell-surface maintenance of Hh protein. MicroRNAs (miRNAs) are a group of widely expressed 21–23 nucleotides non-coding RNAs that repress gene function through interactions with target mRNAs. Here, we have identified a novel miRNA, miR-932, as an important regulator for Boi. We show that overexpression of miR-932 in the wing disc can enhance Hh signaling strength, but reduce its signaling range, a phenotype similar to that of *boi* knockdown. In both in vivo sensor assay and in vitro luciferase assay, miR-932 can suppress Boi by directly binding to its 3′UTR. Meanwhile, down-regulation of miR-932 by sponge elevates the protein level of Boi, confirming that miR-932 is an in vivo regulator of Boi expression. Further, we demonstrate that miR-932 can block Hh signaling when co-expressed with *ihog-RNAi*. Moreover, we find that other predicted miRNAs of Boi fail to suppress it as strong as miR-932. Taken together, our data demonstrate that miR-932 can modulate Hh activity by specifically targeting Boi in *Drosophila*, illustrating the important roles of miRNAs in fine regulation of the Hh signaling pathway.

Introduction

Hedgehog (Hh) is a highly conserved secreted signaling protein that regulates the growth and patterning of many organs in fly and vertebrates (Ingham et al., 2011; Jiang and Hui, 2008; Strigini and Cohen, 1997). In the *Drosophila* wing imaginal disc, Hh is expressed in posterior (P) compartment cells and transported to anterior (A) compartment cells to transduce signaling. Hh binds to Patched (Ptc) and releases the inhibition on Smoothened (Smo). And then activated Smo forms a cytoplasmic signaling complex with two other components, Cos2 and Fu, to transduce signaling via the transcription factor Ci, which subsequently turns on the expression of target genes (Denef et al., 2000; Jia et al., 2003; Lum and Beachy, 2004; Lum et al., 2003b). Secretion, distribution and reception of Hh signals must be tightly regulated, and abnormal Hh signaling is associated with many developmental defects and cancers (Jiang and Hui, 2008; Taipale and Beachy, 2001). Understanding of precise mechanisms of Hh gradient formation will help us to find new strategies for the diagnosis and therapeutic treatment of cancers.

Ihog (Interference hedgehog) and Boi (Brother of ihog) are discovered as components of the Hh signaling (Lum et al., 2003a). As co-receptors of Ptc, they are redundantly required for high-affinity Hh binding and signaling response (Camp et al., 2010; Yan et al., 2010; Yao et al., 2006; Zheng et al., 2010). Ihog and Boi are single transmembrane proteins with four extracellular IgG domains, two extracellular fibronectin (Fn) domains and an intracellular tail. The first Fn domain is required and sufficient for direct binding to HhN (McLellan et al., 2006; Yao et al., 2006), while the second Fn domain is essential for physical interaction between Ihog/Boi and Ptc on the cell surface (Zheng et al., 2010). Cdo and Boc are two closest vertebrate homologs of Ihog and Boi

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which were reported to positively regulate myogenic differentiation (Kang et al., 1998,2002). Further studies demonstrated that they are Sonic Hh binding proteins essential for SHh signaling (Allen et al., 2011; Izzi et al., 2011; Okada et al., 2006; Tenzen et al., 2006).

microRNAs (miRNAs) are small noncoding RNAs that act as post-transcriptional repressors by base-pairing to the 3′ untranslated region (UTR) of their cognate mRNAs (Bushati and Cohen, 2007; Kloosterman and Plasterk, 2006). Their widespread and important roles in animals are highlighted by recent estimates that up to 30% of all genes are targets of miRNAs (Lewis et al., 2005; Stark et al., 2005; Xie et al., 2005). However, the physiological functions of many individual miRNAs remain largely unknown. Combined with computational methods, genetic approaches using model organisms such as *Drosophila* have been used to examine the biological roles of miRNAs at both the organismal and molecular levels (Smibert and Lai, 2008). The first identified miRNA in *Drosophila* is *bantam*, which controls cell proliferation and regulates the pro-apoptotic gene *hid* (Brennecke et al., 2003; Hipfner et al., 2002). Since then, hundreds of miRNAs have been identified, and their biological functions in multiple important signaling pathways have been characterized. For example, expression of miR-315 activates the Wingless pathway by targeting the negative regulators Axin and Notum (Silver et al., 2007). microRNA-9a ensures the precise specification of sensory organ precursors by regulating the pro-neural transcription factor Senseless (Li et al., 2006). miR-8 directly suppresses Notch ligand Serrate to inhibit Notch-induced overgrowth and tumor metastasis (Vallejo et al., 2011). miR-279 directly represses STAT to regulate border cell migration during oogenesis in *Drosophila* (Yoon et al., 2011).

Here, we report the identification of a novel microRNA (miR-932), which acts as a modulator of Hh signaling. Overexpression of miR-932 promotes Hh signaling strength while reducing signaling range. Importantly, we provide the evidence that miR-932 controls Hh signaling by regulating *boi*. Overexpression of miR-932 represses Boi protein level by directly binding to its 3′UTR, while knockdown miR-932 by sponge increases Boi expression. Moreover, we showed that miR-932 exhibited stronger activities in repressing *boi* than several other candidate miRNAs predicted by computational methods (Enright et al., 2003). Taken together, our findings suggest that miR-932 is required for the precise control of Hh signaling in *Drosophila* development.

Material and methods

Drosophila genetics

All stocks were maintained and crossed at 25 °C according to the standard procedures. The *en-Gal*4, *ap-Gal*4, *dpp-Gal*4 *and ywflp; act > y+ > Gal*4, *UAS-GFP* lines were obtained from Bloomington stock center. The *UAS-boi-RNAi and UAS-ihog-RNAi* lines were described in our previous paper (Yan et al., 2010). The *UAS-GFP-GPI-miR-932-sponge*, *UAS-GFP-GPI-miR-981*, *UAS-GFP-GPI-miR-314*, *UAS-GFP-GPI-miR-79*, *UAS-GFP-GPI-miR-4*, *UAS-GFP-GPI- miR-929*, *UAS-GFP-GPI-miR-1014* and *UAS-GFP-GPI-miR-956* transgenic flies were generated using the PhiC31 integrase-mediated site-specific transgenesis system. The *UAS-boi*, *UAS-DsRed-miR-932*, *miR-932-sensor*, *tub-EGFP* and *tub-EGFP-boi-3′UTR* flies were generated by P-element transformation. All transgenic flies were mapped using standard methods.

Plasmid construction

To generate the pUAST-DsRed-miR-932 construct, 800 bp of genomic DNA surrounding miR-932 was amplified by PCR and cloned downstream of DsRed into the pUAST vector. PCR primers are:

forward, 5′-CGGGGTACCCATTTGATTGCGTTCCG-3′;
reverse, 5′-GCTCTAGAGGACAGTTTGGTCTTCG-3′.

A similar strategy was used to make the pWALIUM10-moe-GFP-GPI-miRNA constructs, PCR fragments containing miRNAs were cloned downstream of GFP-GPI in the XbaI site of the pWALIUM10-moe vector (Ni et al., 2011). PCR primers are:

miR-981 forward, 5′-GCTCTAGAGCTGCTGAGCACATTTCCGTTA-3′;
miR-981 reverse, 5′-GCTCTAGAGGTTTGGATTTACAAGCATGATC-3′.
miR-314 forward, 5′-GCTCTAGAGGCGAAACCTCCTACAACCCA-3′;
miR-314 reverse, 5′-GCTCTAGATGGTGGGGCCAAGTGGTAAAC-3′.
miR-79 forward, 5′-GCTCTAGAAGTCCTGGCACGCGTTTGACC-3′;
miR-79 reverse, 5′-GCTCTAGAGGCGAGCATATCTCCAGGGCAG-3′.
miR-4 forward, 5′-GCTCTAGAGATGCATCTTGTGCAGTTATGT-3′;
miR-4 reverse, 5′-GCTCTAGAACAGCCACTGTGATATAGATATG-3′.
miR-929 forward, 5′-GCTCTAGATACCTCGTCACTTACACAGG-3′;
miR-929 reverse, 5′-GCTCTAGAGCAGTATATAGATGCCACTA-3′.
miR-1014 forward, 5′-GCTCTAGATGTCTCCAATTGCTACGAGG-3′;
miR-1014 reverse, 5′-GCTCTAGAGCTGGCCATTCCACTGATGA-3′.
miR-956 forward, 5′-GCTCTAGATGACCAGCTAGGACTAGCAGC-3′;
miR-956 reverse, 5′-GCTCTAGACGTTGTCATGCGTATATGATTA-3′.

The tub-EGFP-boi-3′UTR was generated by cloning a 766 bp fragment of *boi* 3′UTR downstream of pCaSpeR-tub-EGFP (Pek et al., 2009) (a gift from T. Kai), through NotI and XhoI sites. PCR primers are:

forward, 5′-TAGCGGCCGCAGCACAACGAGGAGAAATAT-3′
reverse, 5′-GACTCGAGAGTTTAGGATGCTTCTCTCT-3′

To generate the miR-932-sensor construct, the following primers are annealed (95 °C for 5 min, then slowly cooled down to room temperature) in the annealing buffer (10 nM Tris-HCl, pH=7.5, 100 mM NaCl, 1 mM EDTA), and then directly cloned into the pCaSpeR-tub-EGFP vector through NotI and XhoI sites. PCR primers are:

forward, 5′-GGCCGCCTGCAATGCACTACGGAATTGAAATCA-CACCTGCAATGCACTA CGGAATTGAC-3′
reverse, 5′-TCGAGTCAATTCCGTAGTGCATTGCAGGTGTGATTT-CAATTCCGTAGTGC ATTGCAGGC-3′

To generate the miR-932-sponge construct, the following primers are annealed in the annealing buffer, and then directly cloned into the pWALIUM10-moe-GFP-GPI through NdeI and XbaI sites. PCR primers are:

forward, 5′-TATGCTGCAATGCACATGGGAATTGAGGCTAGCCCTG CAATGCACATGG GAATTGAGGCTAGCCCTGCAATGCACATGGGAATT GAGGCTAGCCCTGCAATGCACATGGGAATTGAGGCTAGCCCTGCAATGC ACATGGGAATTGAGGCTAGCCCTGCAATGCACATGGGAATTGAT-3′
reverse, 5′-CTAGATCAATTCCCATGTGCATTGCAGGGCTAGCCTCA ATTCCCATGTG CATTGCAGGGCTAGCCTCAATTCCCATGTGCATTGCAG GGCTAGCCTCAATTCCCATGTGCATTGCAGGGCTAGCCTCAATTCCCATG TGCATTGCAGGGCTAGCCTCAATTCCCATGTGCATTGCAGCA-3′

Luciferase assay

For validation of miRNAs that targets *boi* 3′UTR, a 766-bp fragment was amplified by PCR from wild-type genomic DNA and cloned downstream of Renilla luciferase in the psiCheck-2 vector (Promega). PCR primers used are:

forward, 5′-GGTTTAAACAGCACAACGAGGAGAAATAT-3′;
reverse, 5′-TTGCGGCCGCAGTTTAGGATGCTTCTCTCT-3′.

boi 3′UTR mutants were generated by changing the seed-binding sites of different miRNAs to GGCGCGCC. Transfections were performed in 24-well plates by using Effectene transfection reagent in S2 cells. In each well, 1 ug of total DNA was added. After 48 h, cells were lysed in passive lysis buffer, dual luciferase assays were carried out (Promega), and the results were analyzed on the luminometer.

Whole-mount staining and microscopy

Fixation and antibody staining in imaginal discs were performed as described (Belenkaya et al., 2004). Primary antibodies used for the immunostainings were: mouse anti-Ptc (DSHB, Apa-1, 1:40), rat anti-Ci (DSHB, 2A1, 1:10), mouse anti-Smo (DSHB, 20C6, 1:50), mouse anti-Fused (DSHB, 22F10, 1:50) and mouse anti-Cos2 (DSHB, 17E11, 1:5), rabbit anti-Col (self-made, 1:100), mouse anti-Boi (self-made, 1:100), rat anti-Ihog (gift from P. Beachy, 1:100). The primary antibodies were detected by fluorescent-conjugated secondary antibodies from Jackson ImmunoResearch Laboratories, Inc. Confocal images were collected using Zeiss 780 confocal microscope with 40X/1.30 oil objectives. Adult wing images were obtained using a Zeiss Axio Imager Z2 microscope. Images were processed using Adobe Photoshop. For quantification of confocal images, the raw data were exported in tiff format. The plot values were measured from selected regions using Image *J* (NIH).

Generation of anti-Boi and anti-Col antibodies

The 346–483Aa coding sequences of Boi and the full length cDNA of Col were cloned into the pGEX-4T-1 vector. The GST fusion proteins were expressed in BL21 E. coli cells and purified using Sigma glutathione-agarose (G4510). The antisera were subsequently affinity purified with Sigma protein G beads (PURE1A).

Bioinformatics

The miRanda (http://www.microrna.org) and TargetScan (http://www.targetscan.org) were used to identify Boi as a conserved miR-932 target, and to search miRNA candidates for *boi* 3′UTR. Sequence alignment of miR-932 and the *boi* 3′UTR across 7 *Drosophilia* genomes was obtained from the University of California at Santa Cruz Genome Browser (http://genome.ucsc.edu). The DeepBase (http://deepbase.sysu.edu.cn/ browseExpress.php) was used to display the tissue expression profiles of four miRNAs.

Results

Identification of miR-932 as an Hh signaling modulator

Pattern formation of *Drosophila* wing is a well-established model system for studying the Hh signaling pathway. During *Drosophila* wing development, Hh is produced by P cells and

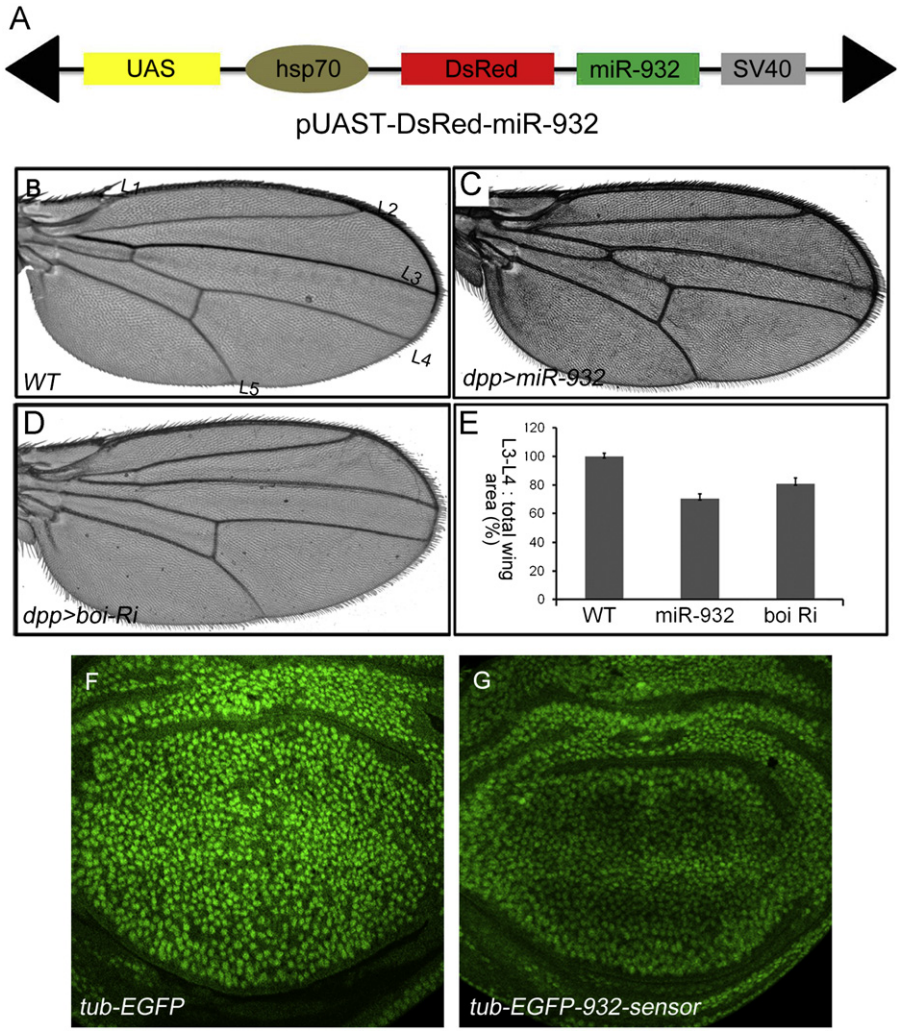

Fig. 1. Overexpression of miR-932 suppresses Hh signaling in *Drosophila* wing. (A) Schematic representation of the pUAST-DsRed-miR-932 construct. (B–D) Compared with adult wing of wild-type fly (B), overexpression of miR-932 causes reduction of the intervein region between L3 and L4 (C), which is similar to knockdown of *boi* expression by RNAi (D). (E) Adult wings were quantified by measuring the area between L3 and L4 space and the whole wing ($n=50$). Results were expressed as a ratio of two areas (F and G). The uniform decrease of the GFP level in miR-932-sensor (G) compared with the tub-EGFP control (F) indicates that miR-932 is uniformly expressed in the wing imaginal disc. The pictures shown in F and G were obtained with a similar laser setting on the confocal microscope.

activates the expression of the high-threshold targets Patched (Ptc) and Collier (Col; Knot), and stabilizes another low-threshold target, Cubitus interruptus (Ci) in the adjacent A cells (Hooper, 2003; Lum and Beachy, 2004). Activation of Hh signaling in these anterior cells is required for patterning the intervein region between L3 and L4 (Mohler et al., 2000). Inhibition of Hh signaling will result in the reduced intervein region between L3 and L4. Recently, we performed a miRNA screening in *Drosophila* to identify new Hh signaling regulators. One of them is miR-5 which can suppress Hh signaling by directly targeting Smo (Wu et al., 2012). Overexpression of another miRNA, miR-932 by *dpp-Gal4* along the A/P boundary causes an obvious reduction of the intervein region between L3 and L4 (Fig. 1C and E), suggesting that miR-932 might also modulate Hh signaling.

To test whether miR-932 is indeed expressed in wing disc, we generated the miR-932-sensor line. Two complementary sites with perfect matches for miR-932 were placed downstream of EGFP controlled by the ubiquitously expressed tubulin (tub) promoter. In this sensor line, miR-932 is expected to bind to the complementary sequence and block the translation of EGFP mRNA and thereby decrease the level of EGFP. To verify the sensor line, we induced miR-932 expression in the *dpp* expression domain along the A/P boundary of the wing disc using *dpp-Gal4* driver. The EGFP expression level is dramatically reduced in the sensor line (Fig. S1B and B′), indicating that the sensor line can be used to investigate the expression profiles of miR-932. Compared with the tub-EGFP control line (Fig. 1F), the miR-932-sensor line shows a uniformly decreased EGFP expression level (Fig. 1G). This result indicates that miR-932 is uniformly expressed in the wing imaginal disc.

To further determine the effect of miR-932 on Hh signaling, we examined the expression of Hh target genes. In wild-type discs, the levels of these target genes are virtually identical in the dorsal (*D*) and ventral (*V*) compartments (Fig. 2A, D and G). Ectopic expression of miR-932 in D compartment by *ap-Gal4* caused elevated levels of Ptc, Col and Ci in cells closer to the *P* compartment; however, the expression ranges of these targets were narrowed (Fig. 2B, E and H). Together with the morphological defects of the wings expressing miR-932, these data suggest further that miR-932 is involved in regulating the Hh signaling in the wing disc.

miR-932 directly targets boi 3′UTR

Computational approaches have been valuable tools in understanding the biology of miRNAs (Bentwich, 2005; Bentwich et al., 2005; Rajewsky, 2006). It is known that the functional miRNA-target site pairing often shows evolutionary conservation (Stark et al., 2003). miR-932 is highly conserved across the 12 sequenced *Drosophila* species. The pre-miR-932 hairpin exhibits stronger conservation than flanking genomic sequences and its stem sequence is much more conserved than the terminal loop (Fig. 3A). To further understand the molecular function of miR-932, we searched for potential miR-932 targets using computational target prediction tools miRanda and TargetScan (Enright et al., 2003; Ruby et al., 2007). One predicted target of miR-932 is Boi. The *boi* 3′UTR contains a putative miR-932 binding site that is conserved in closely related *Drosophila* species, including distantly related *D. yakuba* (Fig. 3B and C). This highly conserved binding site suggests further that Boi might be a target of miR-932 in *Drosophila*.

To determine whether miR-932 inhibits Boi expression in vivo, we performed an immunostaining assay in the wing disc. Endogenous Boi, recognized by Boi antibody (Fig. S2), is expressed ubiquitously throughout the wing pouch (Fig. 4A and C). Ectopic expression of miR-932 at the D compartment using *ap-Gal4*

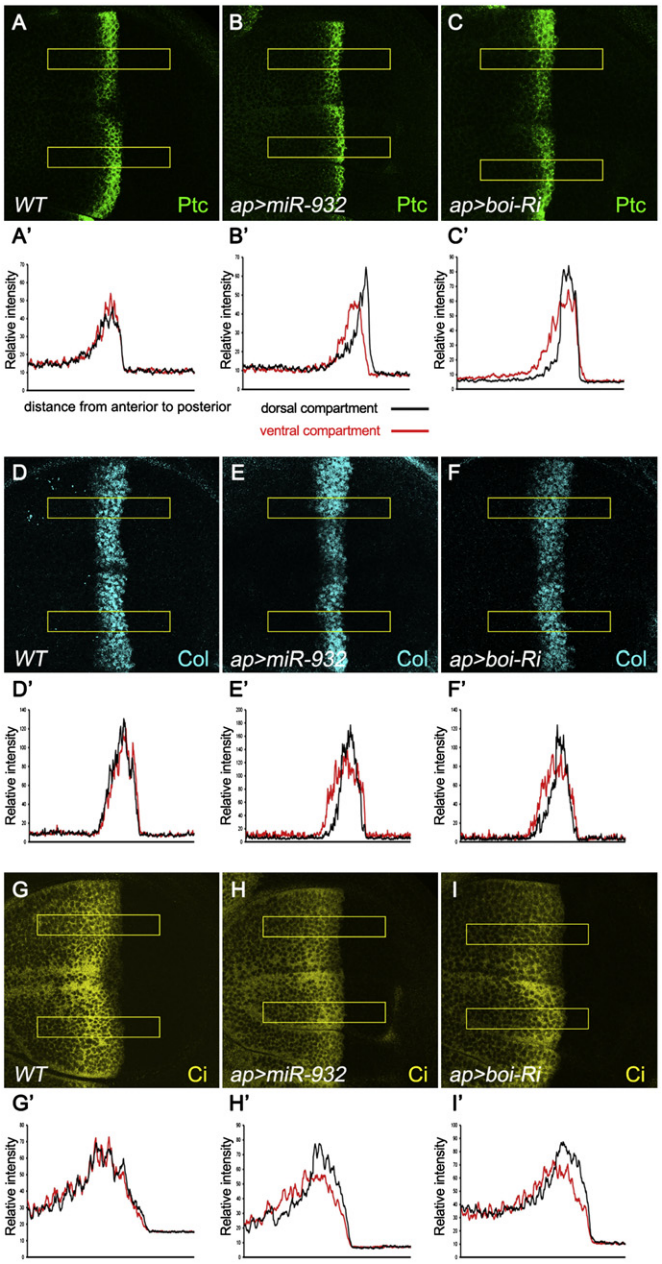

Fig. 2. miR-932 expression enhances Hh signaling strength while reducing signaling range. (A–I) Ptc, Col and Ci staining in *Drosophila* third instar larva wing discs, oriented ventral up and anterior left. (A, D and G) Wild–type discs. (B, E and H) Expression of miR-932 induced in the wing disc by *ap-Gal4* leads to enhanced Hh signaling strength but reduced signaling range. (C, F and I) Knockdown *boi* by RNAi generated similar phenotype. The fluorescence intensities from selected areas (boxed) in these images are quantified in A′–I′. The *y*-axis indicates relative fluorescence intensity and the *x*-axis indicates the distance from the anterior (left) to the posterior (right) compartment of the wing discs.

inhibited the expression of Boi in both A and P compartments (Fig. 4B and B″). Similarly, a dramatic reduction of Boi was also observed at the P compartment when miR-932 was expressed by *en-Gal4* (Fig. 4D and D″). Importantly, knockdown Boi by RNAi using *dpp-Gal4* caused an obvious reduction of the intervein region between L3 and L4, which is similar to overexpression of miR-932 (Fig. 1D and E). Consistently, induction of *ihog* RNAi in the D compartment also enhanced Ptc, Col and Ci expression levels, but reduced their ranges (Fig. 2C, F and I). These data indicate that miR-932 can suppress Boi protein level in vivo.

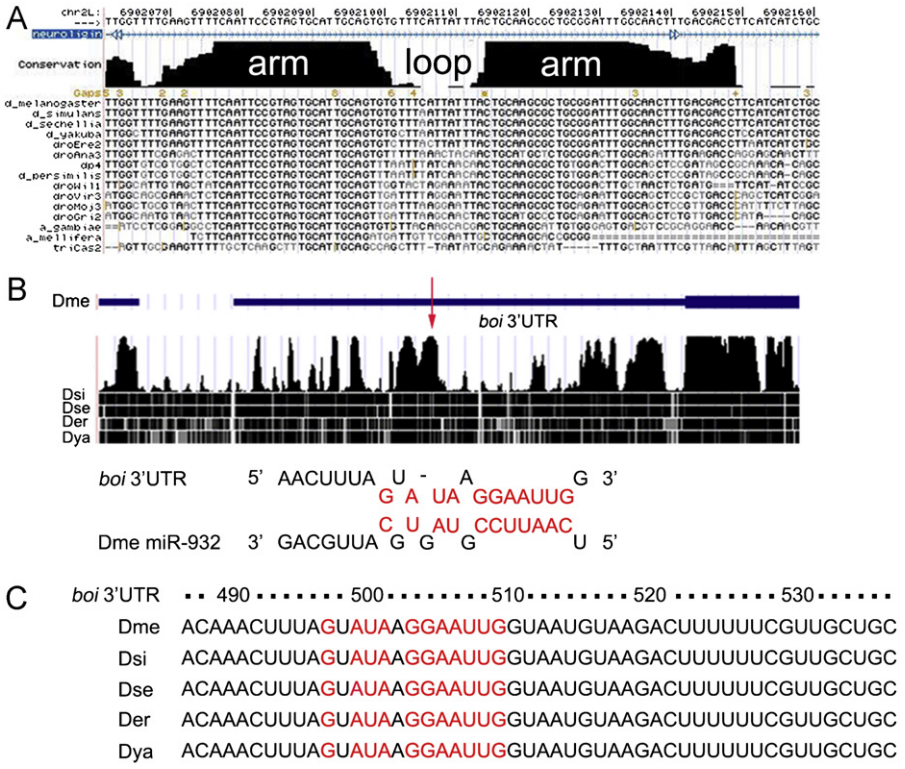

Fig. 3. Boi is predicted as a potential target of miR-932. (A) Alignment of the miR-932 region across 12 Drosophilids. The hairpin arms are much more conserved than the terminal loop and flanking sequences. Alignments and conservation data were produced by the UCSC Genome Center. (B) The predicted miR-932 binding site (top) is highly conserved within the 3'UTR of *boi* mRNAs from different *Drosophila* species (bottom). (C) Conservation of miR-932 seed matches in the *boi* 3'UTR. The perfectly conserved seed sequence (positions 2–8) is indicated by the red box.

To further test the function of the miR-932 targeting site in *boi* 3′UTR, we performed in vivo sensor assay in wing disc and in vitro luciferase assay in *Drosophila* S2 cells. When expressed in the P compartment by *en-Gal4*, miR-932-DsRed repressed specifically and robustly the tub-EGFP-*boi* 3′UTR transgene (Fig. 4F and F′), but did not affect the tub-EGFP control transgene (Fig. 4E and E′). We also generated reporter constructs with the luciferase coding sequence fused to the full-length 3′UTR of *boi* or its mutant form in which the miR-932 targeting site was mutated. miR-932 markedly repressed the activity of the *boi* 3′UTR fused luciferase, while mutation of the seed region (base pairs 2–8) in the putative miR-932 binding site partially blocked the inhibitory effect of miR-932 (Fig. 4G and H). Together, these findings suggest that miR-932 modulates the Hh signaling by directly targeting *boi* 3′UTR.

Blocking the access of miR-932 to its targeting sites increased Boi levels

To further investigate the precise requirement of miR-932 on Boi expression and Hedgehog signaling, we used the microRNA sponge technology (Loya et al., 2009) to generate a UAS-miR-932-sponge (miR-932-SP) transgene. The miR-932-SP contains 6 repetitive sequences complementary to miR-932 with mismatches at the position 9–12 for enhanced stability (Ebert et al., 2007). To test the blocking efficiency of miR-932-SP, we performed the luciferase assay and found miR-932 can antagonize the inhibitory effect of miR-932 on *boi* (Fig. S3). Meanwhile, blocking the miR-932 function using *ap-Gal4* driven UAS-miR-932-SP caused increased Boi protein level in the *D* compartment (Fig. 5A and A″). These results indicate that the miR-932-SP can efficiently compete with the endogenous miR-932 targeting sites, including the ones in *boi* 3′UTR, and subsequently release the inhibition of Boi expression caused by miR-932. Many miRNAs can directly but only mildly repress the expression of in vivo target genes. Similarly, while we observed increased Boi level in miR-932-SP expressing cells, Hh signaling was not significantly altered as indicated by the expression of target genes (Fig. 5B and D″), possibly due to subtle roles of miR-932 on Boi protein.

Together with ihog-RNAi, miR-932 can block Hh signaling transduction

Previous studies have demonstrated that Boi and Ihog are functionally redundant in Hh signaling (Camp et al., 2010; Yan et al., 2010; Zheng et al., 2010). Hh signaling determined by Ptc level is maintained in *boi* or *ihog* knockdown cells (Fig. 6A and A″ and B and B″), but is diminished when both Ihog and Boi are depleted by RNAi (Fig. 6E and E″). To further examine whether miR-932 can regulate the Hh signaling response, we generated miR-932 overexpression clones adjacent to the A/P boundary. Ptc expression was only mildly reduced in miR-932 overexpression clones (Fig. 6C and C″), but was obviously decreased in miR-932 overexpression clones when Ihog was also removed by RNAi (Fig. 6D and D″). Together, our results argue that miR-932 can regulate Hh signaling by targeting Boi in the wing disc.

miR-932 cannot suppress other Hh signal transducers

It has been known that an individual miRNA can control multiple biological processes by targeting different genes. For example, miR-184 controls germline stem cell differentiation by tuning the Dpp receptor Saxophone, D/V patterning of the egg shell by regulating the gurken transport factor K10, and A/P patterning of the blastoderm by tuning the transcriptional repressor Tramtrack69 (Iovino et al., 2009). Moreover, individual

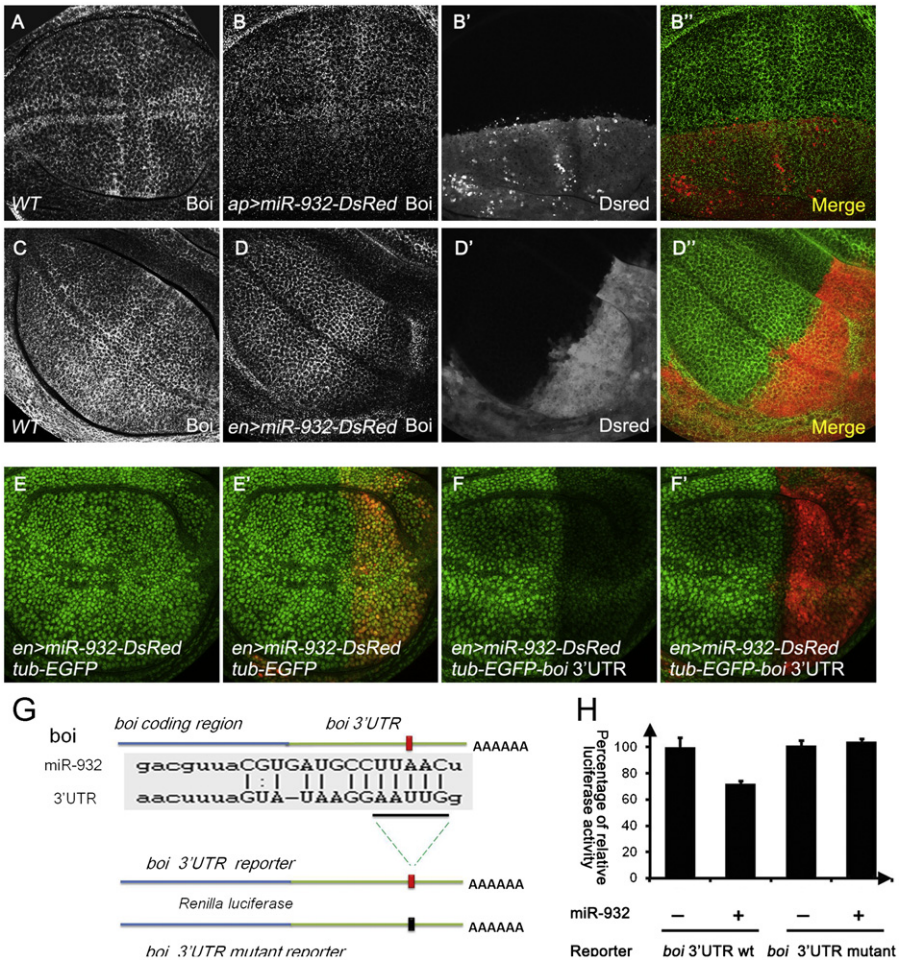

Fig. 4. miR-932 expression suppresses Boi expression level. (A–D″) Boi antibody staining in wing discs. Compared with the wild-type disc (A and C), ectopic expression of miR-932 in the dorsal compartment by *ap-Gal4* (B–B″) causes a strong reduction of Boi expression. Overexpressing miR-932 in the posterior compartment by *en-Gal4* (C–C″) also dramatically inhibits Boi expression. (E–F′) Cells that express miR-932-DsRed under the control of *en-Gal4* did not affect the expression of a control tub-GFP sensor (E and E′), but strongly inhibited sensor fused to the 3'UTR of *boi* (F and F′). (G) The *boi* 3'UTR containing one conserved 7-mer (red box) miR-932 target site was fused to a firefly luciferase reporter construct. The *boi* 3'UTR mutant containing mismatched nucleotides (green) is shown at the bottom of the panel. (H) Luciferase assay for miR-932. miR-932 inhibits the expression of a *boi* 3'UTR-luciferase reporter in cultured *Drosophila* S2 cells, but the mutant construct was immune to miR-932. The ratio of Renilla:firefly activity for each series was normalized to the response of the empty psiCHECK2 sensor, whose baseline ratio was set to 100.

miRNAs can also simultaneously suppress several components of the same signaling pathway. For example, *Drosophila* miR-8 inhibits the Wingless pathway by repressing Wntless, TCF and CG32767 (Kennell et al., 2008) while human miR-324-5p acts as a suppressor of the Hh pathway by targeting both Smoothened and transcription factor Gli1 (Ferretti et al., 2008).

To determine whether miR-932 is able to regulate other Hh signaling components, we examined their protein levels in miR-932 expressing cells. Ihog protein is expressed ubiquitously throughout the wing disc (Fig. 7A). Ihog levels were not reduced in miR-932 expressing cells at the *D* compartment driven by *ap-Gal4* (Fig. 7E and E″). In the wild type discs, the protein level of Smo in the P compartment is higher than that in the A compartment (Fig. 7B), while Fu and Cos2 are expressed more in the A compartment than in the P compartment (Fig. 7C and D). When miR-932 expression was induced in the D compartment, the levels of Smo was not reduced in both A and P compartments (Fig. 7F F″). Interestingly, the levels of Fu and Cos2 were even slightly increased in both A and P compartments (Fig. 7G G″ and H-H″), which might be due to the indirect effect of miR-932. These results indicate that miR-932 mudulates Hh signaling by suppressing Boi, but not other Hh signaling components.

Comparison of miR-932 with other predicted miRNAs in repressing Boi expression

Several miRNAs are collectively predicted to target Boi using the targeting prediction databases. There are seven additional miRNAs which target *boi* 3′UTR and are conserved in 12 *Drosophila* species, including miR-4 (one site), miR-79 (one site), miR-314 (two sites), miR-929 (one site), miR-956 (one site), miR-981 (one site) and miR-1014 (one site). Among them, miR-314 and miR-1014 have higher scores than miR-932 according to prediction algorithms (Enright et al., 2003).

To test whether these miRNAs were able to suppress Boi, we overexpressed them in the wing disc and detected the Boi protein level by antibody staining. Overexpression of miR-4, miR-79 and miR-981 cause strong morphological change of wing disc, even they are derived by *ap-Gal4-Gal80^{ts}*. So it is very difficult to determine whether these miRNAs have effects on Boi expression by antibody staining experiment. Induced miR-314, miR-929, miR-956 and miR-1014 in the dorsal compartments by *ap-Gal4* cannot suppress Boi as strong as miR-932 (Fig. 8A and D′). Similarly, we found that these miRNAs cannot inhibit the luciferase activity of *boi* 3′UTR reporter as strong as miR-932 in

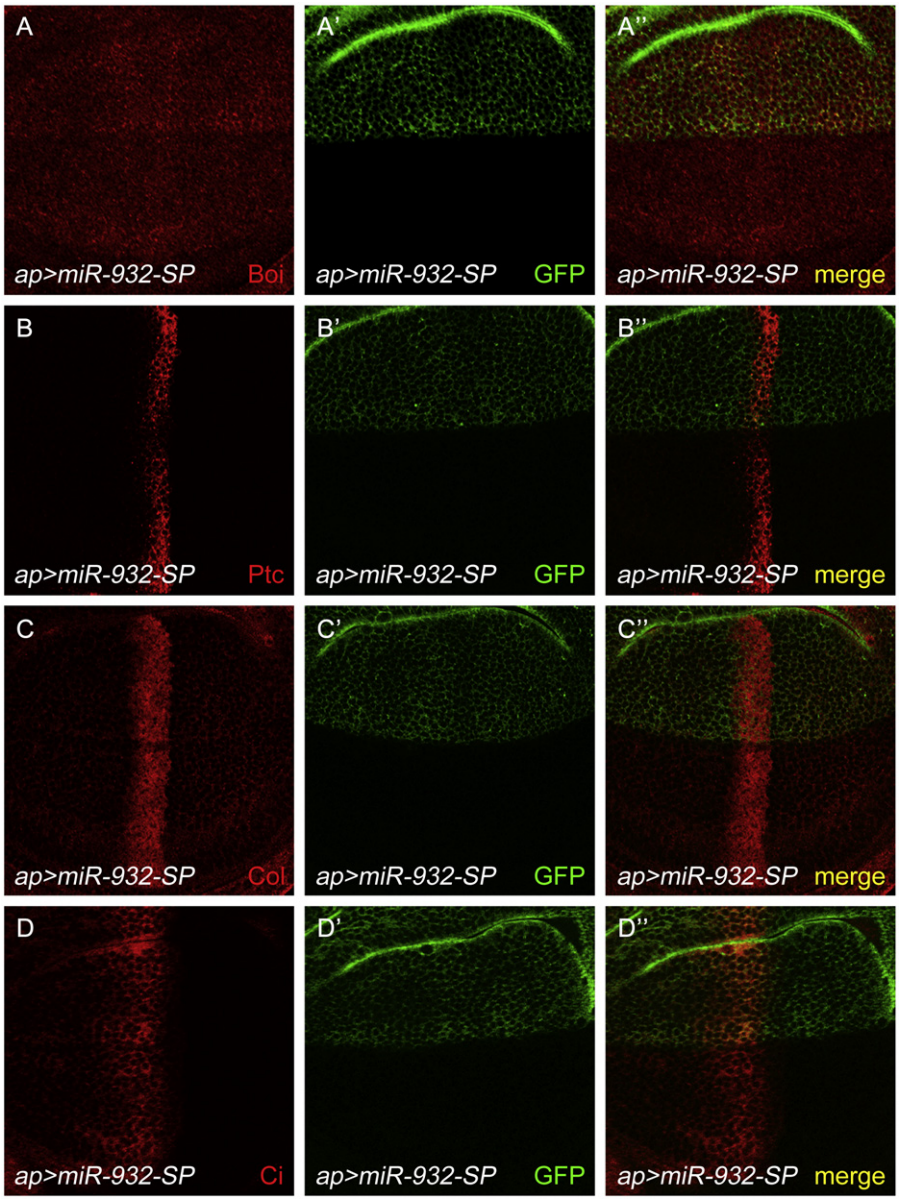

Fig. 5. Downregulation of miR-932 by sponge elevates the protein level of Boi. Expression of miR-932-SP in the dorsal compartment by *ap-Gal4* leads to the increased protein level of Boi (A–A″), but cannot change the protein levels of Ptc (B–B″), Col (C–C″) and Ci (D–D″).

Drosophila S2 cells (Fig. 8E). These observations suggest that, among these predicted miRNAs, miR-932 is a uniquely potent regulator of Boi.

Discussion

A precise control of Hh signaling is important for a wide variety of processes during embryonic development and adult tissue homeostasis (Varjosalo and Taipale, 2008). Inappropriate activity of the Hh signaling pathway contributes to numerous human disorders, especially different types of tumors (Teglund and Toftgard, 2010). A hallmark of Hh signaling is its ability to act over a long range and control distinct cell fates in a concentration dependent manner, raising the important question of how Hh gradients are precisely controlled during development. In this study, we identified a novel miRNA, miR-932, which modulates Hh signaling by directly targeting Hh co-receptor Boi.

Previous work in Zebrafish shows that miR-214 enables precise specification of muscle cell types by sharpening cellular responses to Hh signaling through targeting the negative regulator Su(fu) (Flynt et al., 2007). In a miRNA high-throughput profile screening, miR-125b, miR-326 and miR-324-5p have been found to directly bind to *smo* 3′UTR and inhibit Hh signaling in mammalian cancer cells (Ferretti et al., 2008). miR-12 and miR-283 have been shown to be able to regulate Hh pathway by binding to the 3′UTR of *smo*, *cos2* and *fu* in a *Drosophila* genetic screen (Friggi-Grelin et al., 2008). Similarly, our recent work also identified miR-5 can suppress Hh signaling by directly targeting to Smo (Wu et al., 2012). Together, these observations indicate that microRNA-mediated silencing mechanisms play a critical role in the precise control of Hh signaling.

It is important to note that miR-932 only suppresses Boi but not Ihog, although they are homologues and functionally redundant as co-receptors for Hh signaling. One explanation is that the 3′UTR sequences of *ihog* and *boi* are not evolutionary conserved and share no similarity. Ihog and Boi have individual expression

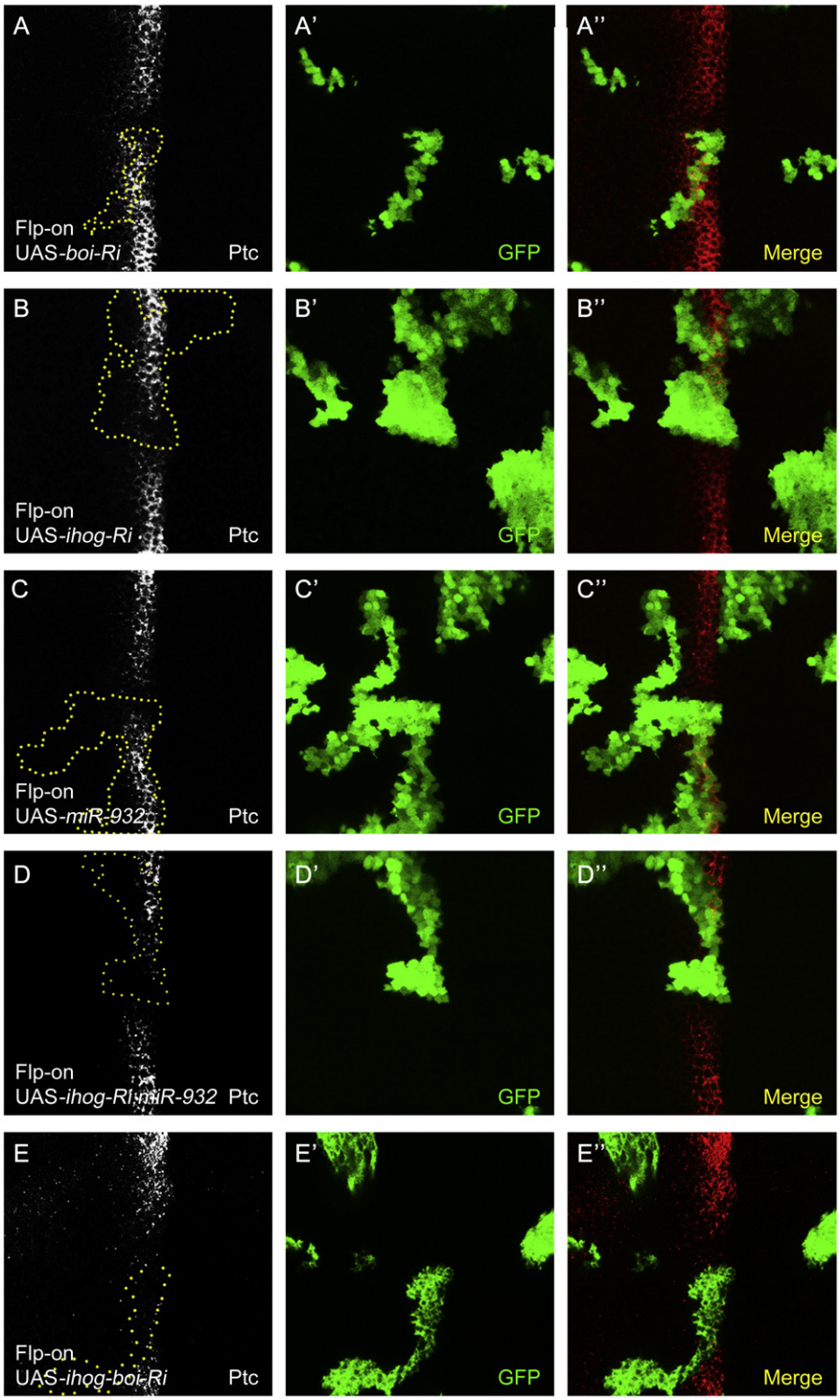

Fig. 6. Together with *ihog-RNAi*, miR-932 can block Hh signaling transduction. Ptc expression is slightly reduced in *boi-RNAi* (A–A″), *ihog-RNAi* clone cells (B–B″) or miR-932 overexpression cells (C–C″), but is significantly reduced in *ihog-RNAi* clone cells that also express miR-932 (D–D″), similarly to the *ihog-boi-RNAi* clone cells (E–E″). All clones are marked by GFP.

patterns and functions in some tissues, and they are likely to be controlled by different mechanisms. For example, Boi limits Hh levels to suppress follicle stem cell proliferation in *Drosophila* ovary, but Ihog mutant does not exhibit any phenotype in this process (Hartman et al., 2010). Unfortunately, miR-932 is not expressed in the Terminal Filament where Boi is expressed and suppresses follicle stem cell proliferation (Fig. 4S). Moreover, the murine homologues, Cdo and Boc, also have been shown to display diverse phenotypes. *Cdo* mutant mice display mild to intermediate forms of holoprosencephaly, a classic manifestation of Hh signaling deficiency (Allen et al., 2007; Cole and Krauss, 2003; Tenzen et al., 2006; Zhang et al., 2006). *Boc* mutant mice show defects in Hh signal-dependent axonal pathfinding by dorsal neurons in the developing neural tube (Okada et al., 2006; Yam et al., 2009). These data indicate that Ihog/Boi and Cdo/Boc have distinct expression patterns and functions in some

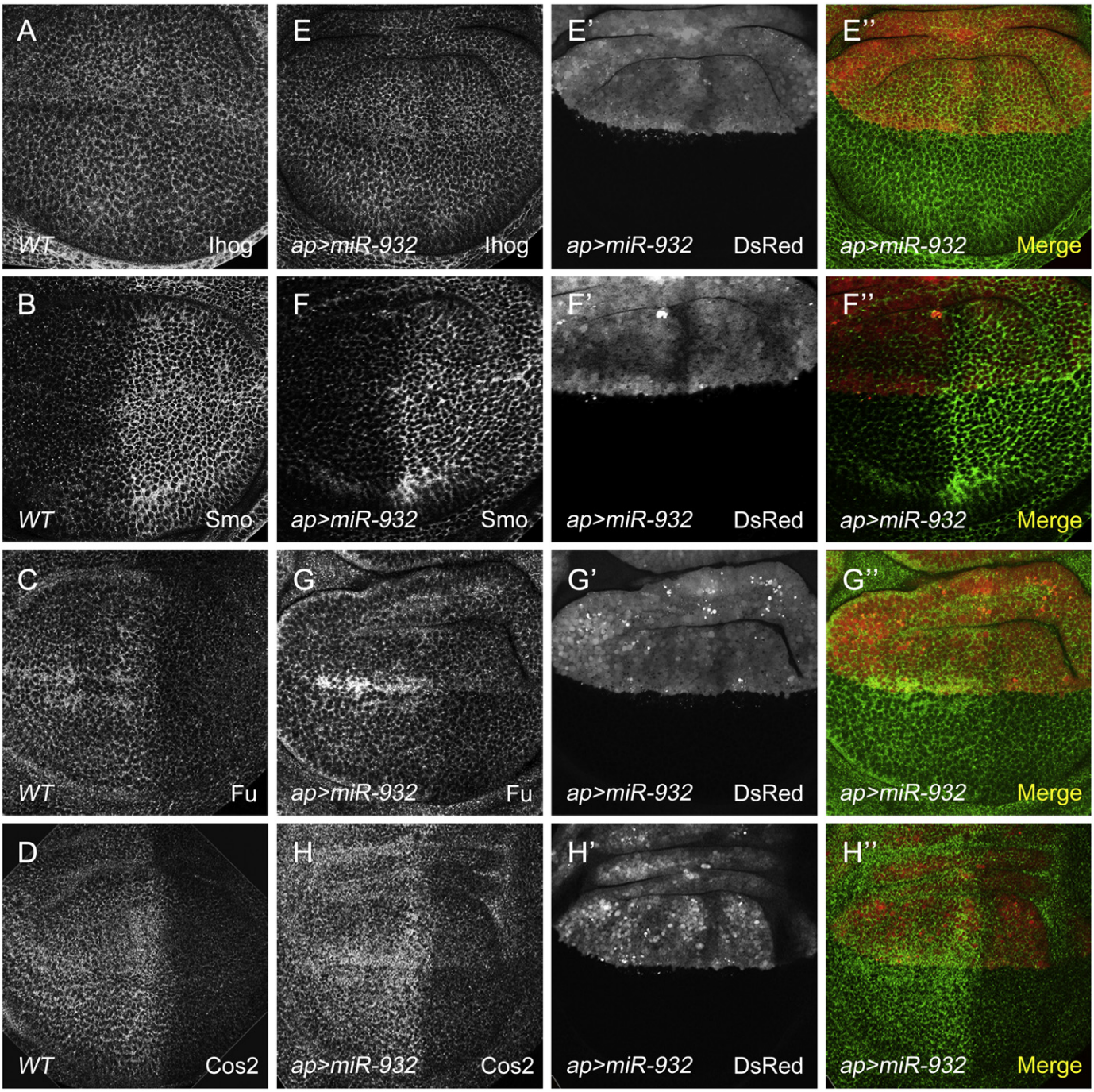

Fig. 7. miR-932 cannot suppress other signal transduction components of Hh pathway. (A–D) Ihog, Smo, Cos2 and Fu staining in wild-type wing discs. (E–H″) Ectopic expression of miR-932 in the dorsal compartment induced by *ap-Gal4* cannot suppress the protein levels of Ihog (E–E″) and Smo (F–F′). The levels of Fu (G–G″) and Cos2 (H–H″) are even slightly increased in miR-932 expressing cells.

tissues. On the basis of our data here, we propose that miRNA mediated mRNA silencing may be one of the mechanisms to regulate the expression of Ihog/Boi and Cdo/Boc during development.

In this work, we overexpressed all eight conserved predicted miRNAs to detect whether they can equally suppress Boi. Surprisingly, except the miR-4, miR-79 and miR-981, only miR-929 can slightly repress Boi expression. Both miR-314 and miR-1014 fails to suppress Boi expression although they have higher scores according to prediction algorithms and miR-314 even has two predicted binding sites on the 3′UTR of *boi*. These observations indicate that the importance of individual miRNAs cannot be simply translated from the existence of conserved miRNA seed matches from available databases. Our data demonstrate that although the bioinformatics methods provide powerful tools for the investigation of the miRNA-mediated gene regulation, the functional analyses are essential for the identification and validation of the biological relevance between miRNA and their predicted targets. A collection of *Drosophila* genome-wide miRNA transgenes has recently been established. It was found that expression of many of these miRNA can induce specific developmental defects in the wing (Bejarano et al., 2012; Schertel et al., 2012; Szuplewski et al., 2012). Further examination of these miRNA will provide novel insights into the regulation of gene expression by miRNAs in development.

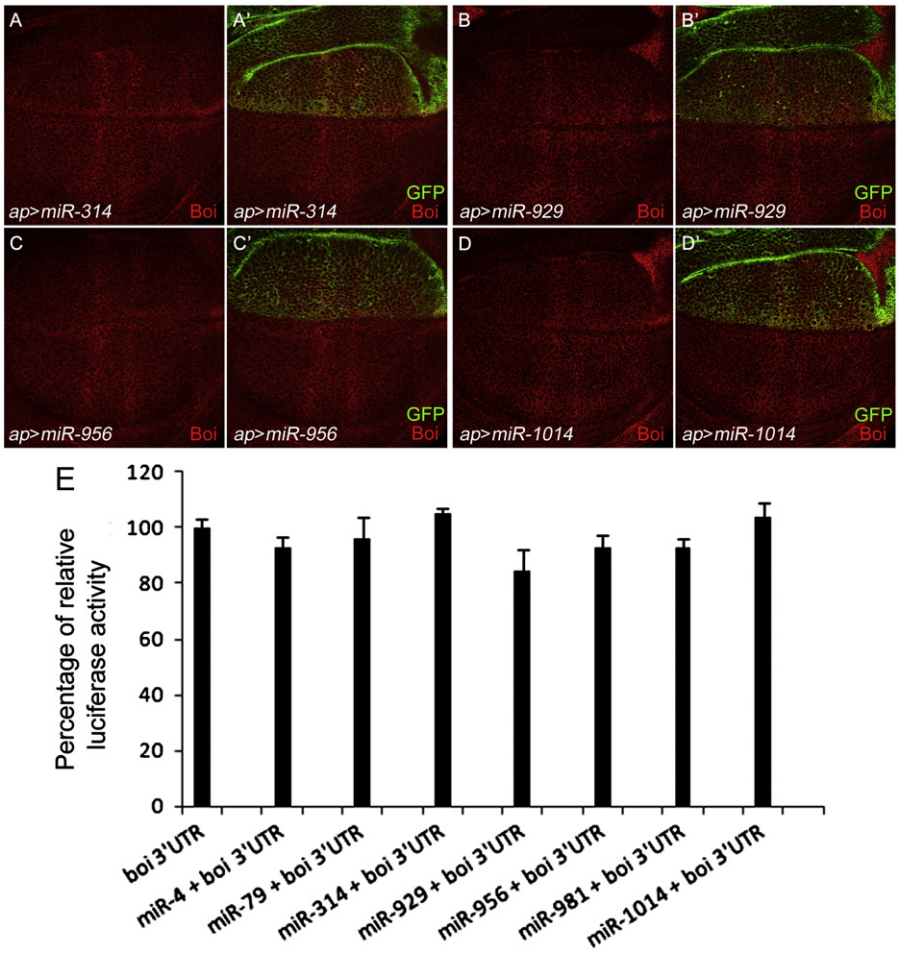

Fig. 8. The suppression effects of other predicted miRNAs on Boi expression. (A–D′) Ectopic expression of miR-314, miR-929, miR-956 and miR-1014 in the posterior compartments by *en-Gal* cannot suppress Boi as strong as miR-932. (E–H) miR-4, miR-79, miR-314, miR-929, miR-956, miR-981 and miR-1014 cannot inhibit the expression of the *boi* 3'UTR-luciferase reporter in cultured *Drosophila* S2 cells.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.02.002.

References

Allen, B.L., Song, J.Y., Izzi, L., Althaus, I.W., Kang, J.S., Charron, F., Krauss, R.S., McMahon, A.P., 2011. Overlapping roles and collective requirement for the coreceptors GAS1, CDO, and BOC in SHH pathway function. Dev. Cell. 20, 775–787.

Allen, B.L., Tenzen, T., McMahon, A.P., 2007. The Hedgehog-binding proteins Gas1 and Cdo cooperate to positively regulate Shh signaling during mouse development. Genes Dev. 21, 1244–1257.

Bejarano, F., Bortolamiol-Becet, D., Dai, Q., Sun, K., Saj, A., Chou, Y.T., Raleigh, D.R., Kim, K., Ni, J.Q., Duan, H., Yang, J.S., Fulga, T.A., Van Vactor, D., Perrimon, N., Lai, E.C., 2012. A genome-wide transgenic resource for conditional expression of Drosophila microRNAs. Development 139, 2821–2831.

Belenkaya, T.Y., Han, C., Yan, D., Opoka, R.J., Khodoun, M., Liu, H., Lin, X., 2004. Drosophila Dpp morphogen movement is independent of dynamin-mediated endocytosis but regulated by the glypican members of heparan sulfate proteoglycans. Cell 119, 231–244.

Bentwich, I., 2005. Prediction and validation of microRNAs and their targets. FEBS Lett. 579, 5904–5910.

Bentwich, I., Avniel, A., Karov, Y., Aharonov, R., Gilad, S., Barad, O., Barzilai, A., Einat, P., Einav, U., Meiri, E., Sharon, E., Spector, Y., Bentwich, Z., 2005. Identification of hundreds of conserved and nonconserved human microRNAs. Nat. Genet. 37, 766–770.

Brennecke, J., Hipfner, D.R., Stark, A., Russell, R.B., Cohen, S.M., 2003. Bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. Cell 113, 25–36.

Bushati, N., Cohen, S.M., 2007. microRNA functions. Annu. Rev. Cell. Dev. Biol. 23, 175–205.

Camp, D., Currie, K., Labbe, A., van Meyel, D.J., Charron, F., 2010. Ihog and Boi are essential for Hedgehog signaling in Drosophila. Neural Dev. 5, 28.

Cole, F., Krauss, R.S., 2003. Microform holoprosencephaly in mice that lack the Ig superfamily member Cdon. Curr. Biol. 13, 411–415.

Denef, N., Neubuser, D., Perez, L., Cohen, S.M., 2000. Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened. Cell 102, 521–531.

Ebert, M.S., Neilson, J.R., Sharp, P.A., 2007. MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. Nat. Method. 4, 721–726.

Enright, A.J., John, B., Gaul, U., Tuschl, T., Sander, C., Marks, D.S., 2003. MicroRNA targets in Drosophila. Genome Biol. 5, R1.

Ferretti, E., De Smaele, E., Miele, E., Laneve, P., Po, A., Pelloni, M., Paganelli, A., Di Marcotullio, L., Caffarelli, E., Screpanti, I., Bozzoni, I., Gulino, A., 2008.

Concerted microRNA control of Hedgehog signalling in cerebellar neuronal progenitor and tumour cells. EMBO J. 27, 2616–2627.
Flynt, A.S., Li, N., Thatcher, E.J., Solnica-Krezel, L., Patton, J.G., 2007. Zebrafish miR-214 modulates Hedgehog signaling to specify muscle cell fate. Nat. Genet. 39, 259–263.
Friggi-Grelin, F., Lavenant-Staccini, L., Therond, P., 2008. Control of antagonistic components of the hedgehog signaling pathway by microRNAs in Drosophila. Genetics 179, 429–439.
Hartman, T.R., Zinshteyn, D., Schofield, H.K., Nicolas, E., Okada, A., O'Reilly, A.M., 2010. Drosophila Boi limits Hedgehog levels to suppress follicle stem cell proliferation. J. Cell. Biol. 191, 943–952.
Hipfner, D.R., Weigmann, K., Cohen, S.M., 2002. The bantam gene regulates Drosophila growth. Genetics 161, 1527–1537.
Hooper, J.E., 2003. Smoothened translates Hedgehog levels into distinct responses. Development 130, 3951–3963.
Ingham, P.W., Nakano, Y., Seger, C., 2011. Mechanisms and functions of Hedgehog signalling across the metazoa. Nat. Rev. Genet. 12, 393–406.
Iovino, N., Pane, A., Gaul, U., 2009. miR-184 has multiple roles in Drosophila female germline development. Dev. Cell. 17, 123–133.
Izzi, L., Levesque, M., Morin, S., Laniel, D., Wilkes, B.C., Mille, F., Krauss, R.S., McMahon, A.P., Allen, B.L., Charron, F., 2011. Boc and Gas1 each form distinct Shh receptor complexes with Ptch1 and are required for Shh-mediated cell proliferation. Dev. Cell. 20, 788–801.
Jia, J., Tong, C., Jiang, J., 2003. Smoothened transduces Hedgehog signal by physically interacting with Costal2/Fused complex through its C-terminal tail. Genes Dev. 17, 2709–2720.
Jiang, J., Hui, C.C., 2008. Hedgehog signaling in development and cancer. Dev. Cell. 15, 801–812.
Kang, J.S., Mulieri, P.J., Hu, Y., Taliana, L., Krauss, R.S., 2002. BOC, an Ig superfamily member, associates with CDO to positively regulate myogenic differentiation. EMBO J. 21, 114–124.
Kang, J.S., Mulieri, P.J., Miller, C., Sassoon, D.A., Krauss, R.S., 1998. CDO, a robo-related cell surface protein that mediates myogenic differentiation. J. Cell Biol. 143, 403–413.
Kennell, J.A., Gerin, I., MacDougald, O.A., Cadigan, K.M., 2008. The microRNA miR-8 is a conserved negative regulator of Wnt signaling. Proc. Natl. Acad. Sci. USA 105, 15417–15422.
Kloosterman, W.P., Plasterk, R.H., 2006. The diverse functions of microRNAs in animal development and disease. Dev. Cell. 11, 441–450.
Lewis, B.P., Burge, C.B., Bartel, D.P., 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120, 15–20.
Li, Y., Wang, F., Lee, J.A., Gao, F.B., 2006. MicroRNA-9a ensures the precise specification of sensory organ precursors in Drosophila. Genes Dev. 20, 2793–2805.
Loya, C.M., Lu, C.S., Van Vactor, D., Fulga, T.A., 2009. Transgenic microRNA inhibition with spatiotemporal specificity in intact organisms. Nat. Methods. 6, 897–903.
Lum, L., Beachy, P.A., 2004. The Hedgehog response network: sensors, switches, and routers. Science 304, 1755–1759.
Lum, L., Yao, S., Mozer, B., Rovescalli, A., Von Kessler, D., Nirenberg, M., Beachy, P.A., 2003a. Identification of Hedgehog pathway components by RNAi in Drosophila cultured cells. Science 299, 2039–2045.
Lum, L., Zhang, C., Oh, S., Mann, R.K., von Kessler, D.P., Taipale, J., Weis-Garcia, F., Gong, R., Wang, B., Beachy, P.A., 2003b. Hedgehog signal transduction via Smoothened association with a cytoplasmic complex scaffolded by the atypical kinesin, Costal-2. Mol. Cell. 12, 1261–1274.
McLellan, J.S., Yao, S., Zheng, X., Geisbrecht, B.V., Ghirlando, R., Beachy, P.A., Leahy, D.J., 2006. Structure of a heparin-dependent complex of Hedgehog and Ihog. Proc. Natl. Acad. Sci. USA 103, 17208–17213.
Mohler, J., Seecoomar, M., Agarwal, S., Bier, E., Hsai, J., 2000. Activation of knot (kn) specifies the 3-4 intervein region in the Drosophila wing. Development 127, 55–63.
Ni, J.Q., Zhou, R., Czech, B., Liu, L.P., Holderbaum, L., Yang-Zhou, D., Shim, H.S., Tao, R., Handler, D., Karpowicz, P., Binari, R., Booker, M., Brennecke, J., Perkins, L.A., Hannon, N., Perrimon, N., 2011. A genome-scale shRNA resource for transgenic RNAi in Drosophila. Nat. Methods. 8, 405–407.
Okada, A., Charron, F., Morin, S., Shin, D.S., Wong, K., Fabre, P.J., Tessier-Lavigne, M., McConnell, S.K., 2006. Boc is a receptor for sonic hedgehog in the guidance of commissural axons. Nature 444, 369–373.
Pek, J.W., Lim, A.K., Kai, T., 2009. Drosophila maelstrom ensures proper germline stem cell lineage differentiation by repressing microRNA-7. Dev. Cell. 17, 417–424.
Rajewsky, N., 2006. MicroRNA target predictions in animals. Nat. Genet. 38, Suppl. S8–13.
Ruby, J.G., Stark, A., Johnston, W.K., Kellis, M., Bartel, D.P., Lai, E.C., 2007. Evolution, biogenesis, expression, and target predictions of a substantially expanded set of Drosophila microRNAs. Genome Res. 17, 1850–1864.
Schertel, C., Rutishauser, T., Forstemann, K., Basler, K., 2012. Functional characterization of drosophila microRNAs by a novel in vivo Library. Genetics 192, 1543–1552.
Silver, S.J., Hagen, J.W., Okamura, K., Perrimon, N., Lai, E.C., 2007. Functional screening identifies miR-315 as a potent activator of wingless signaling. Proc. Natl. Acad. Sci. USA 104, 18151–18156.
Smibert, P., Lai, E.C., 2008. Lessons from microRNA mutants in worms, flies and mice. Cell Cycle 7, 2500–2508.
Stark, A., Brennecke, J., Bushati, N., Russell, R.B., Cohen, S.M., 2005. Animal MicroRNAs confer robustness to gene expression and have a significant impact on 3′UTR evolution. Cell 123, 1133–1146.
Stark, A., Brennecke, J., Russell, R.B., Cohen, S.M., 2003. Identification of drosophila microRNA targets. PLoS Biol. 1, E60.
Strigini, M., Cohen, S.M., 1997. A Hedgehog activity gradient contributes to AP axial patterning of the Drosophila wing. Development 124, 4697–4705.
Szuplewski, S., Kugler, J.M., Lim, S.F., Verma, P., Chen, Y.W., Cohen, S.M., 2012. MicroRNA transgene overexpression complements deficiency-based modifier screens in Drosophila. Genetics 190, 617–626.
Taipale, J., Beachy, P.A., 2001. The Hedgehog and Wnt signalling pathways in cancer. Nature 411, 349–354.
Teglund, S., Toftgard, R., 2010. Hedgehog beyond medulloblastoma and basal cell carcinoma. Biochim. Biophys. Acta. 1805, 181–208.
Tenzen, T., Allen, B.L., Cole, F., Kang, J.S., Krauss, R.S., McMahon, A.P., 2006. The cell surface membrane proteins Cdo and Boc are components and targets of the Hedgehog signaling pathway and feedback network in mice. Dev. Cell. 10, 647–656.
Vallejo, D.M., Caparros, E., Dominguez, M., 2011. Targeting Notch signalling by the conserved miR-8/200 microRNA family in development and cancer cells. EMBO J. 30, 756–769.
Varjosalo, M., Taipale, J., 2008. Hedgehog: functions and mechanisms. Genes Dev. 22, 2454–2472.
Wu, L.F., Gao, L., Hou, X.M., Zhang, Q.H., Li, S., Yang, Y.F., Lin, X.H., 2012. Drosophila miR-5 suppresses Hedgehog signaling by directly targeting smoothened. FEBS Lett. 586, 4052–4060.
Xie, X., Lu, J., Kulbokas, E.J., Golub, T.R., Mootha, V., Lindblad-Toh, K., Lander, E.S., Kellis, M., 2005. Systematic discovery of regulatory motifs in human promoters and 3′ UTRs by comparison of several mammals. Nature 434, 338–345.
Yam, P.T., Langlois, S.D., Morin, S., Charron, F., 2009. Sonic hedgehog guides axons through a noncanonical, Src-family-kinase-dependent signaling pathway. Neuron 62, 349–362.
Yan, D., Wu, Y., Yang, Y., Belenkaya, T.Y., Tang, X., Lin, X., 2010. The cell-surface proteins Dally-like and Ihog differentially regulate Hedgehog signaling strength and range during development. Development 137, 2033–2044.
Yao, S., Lum, L., Beachy, P., 2006. The ihog cell-surface proteins bind Hedgehog and mediate pathway activation. Cell 125, 343–357.
Yoon, W.H., Meinhardt, H., Montell, D.J., 2011. miRNA-mediated feedback inhibition of JAK/STAT morphogen signalling establishes a cell fate threshold. Nat. Cell Biol. 13, 1062–1069.
Zhang, W., Kang, J.S., Cole, F., Yi, M.J., Krauss, R.S., 2006. Cdo functions at multiple points in the Sonic Hedgehog pathway, and Cdo-deficient mice accurately model human holoprosencephaly. Dev. Cell. 10, 657–665.
Zheng, X., Mann, R.K., Sever, N., Beachy, P.A., 2010. Genetic and biochemical definition of the Hedgehog receptor. Genes Dev. 24, 57–71.